BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

Biochemistry **2009,** *48,* 8491–8493 8491
DOI: 10.1021/bi9010539

Arabidopsis thaliana Mitochondrial Glyoxalase 2-1 Exhibits β-Lactamase Activity†

Pattraranee Limphong,‡ George Nimako,‡ Pei W. Thomas,§ Walter Fast,§ Christopher A. Makaroff,‡ and Michael W. Crowder*,‡

‡*Department of Chemistry and Biochemistry, 160 Hughes Hall, 701 East High Street, Miami University, Oxford, Ohio 45056, and* §*Division of Medicinal Chemistry, College of Pharmacy, University of Texas, Austin, Texas 78712*

Received June 22, 2009; Revised Manuscript Received August 14, 2009

ABSTRACT: In an effort to determine the physiological role of *Arabidopsis thaliana* Glx2-1, we attempted to uncover a substrate for the enzyme. Glx2-1 did not effectively process 192 diverse substrates found in a commercial screen used for microorganism identification or exhibit arylsulfatase, lactonase, or phosphotriesterase activities. However, Glx2-1 does exhibit β-lactamase activity with k_{cat}/K_M values from 10^3 to 10^5 M^{-1} s^{-1}. Glx2-1 can hydrolyze cephalosporins and carbapenems, albeit with rate constants slower than those of most metallo-β-lactamases. The potential role of a β-lactamase in the mitochondria of plant cells is briefly discussed.

Enzymes containing the β-lactamase fold are members of a large superfamily of proteins that catalyze a wide variety of enzymatic reactions. The β-lactamase fold consists of an αβ/βα sandwich motif, made up of a core unit of two β-sheets surrounded by solvent-exposed α-helices (*1*). Members of this superfamily contain a conserved HXHXD motif that has been shown to bind Zn(II), Fe, and Mn. There are several enzymes in the β-lactamase fold family, including metallo-β-lactamases, glyoxalase 2, lactonase, rubredoxin:oxygen oxidoreductase (ROO), arylsulfatase, phosphodiesterase, carboxylesterase (*2*), and tRNA maturase (*3*). The prototypical and best-studied members of the β-lactamase fold superfamily are the metallo-β-lactamases (*4*), which are produced by some bacteria and are responsible for bacterial resistance to β-lactam antibiotics.

Glyoxalase 2, Glx2, is the second enzyme in the ubiquitous glyoxalase system, which detoxifies 2-oxoaldehydes from cells. Glx2 hydrolyzes the thioester bond of *S*-D-lactoylglutathione (SLG), which is a product of the glyoxalase 1 (Glx1)-catalyzed isomerization of the thiohemiacetal produced spontaneously from the reaction of glutathione and methylglyoxal. Glyoxalase 2 has been biochemically characterized from many different sources, including plants, mammals, and bacteria (*5*−*9*). A crystal structure of human glyoxalase 2 revealed a tertiary structure that was similar to that previously reported for the metallo-β-lactamases; however, there were some distinct differences in the metal binding site (*10*). The crystallographers concluded that human Glx2 contains a dinuclear Zn(II) site; however, this conclusion has been recently questioned (*11*) (Figure 1).

Sequence similarity searches of the *Arabidopsis thaliana* genome/proteome identified five putative proteins that had the invariant HXHXD motif that were predicted to be Glx2 isozymes (Glx2-1, Glx2-2, Glx2-3, Glx2-4, and Glx2-5) (*12*) (Table S1 of the Supporting Information). The presence of potential N-terminal targeting sequences suggested that Glx2-1, Glx2-4, and Glx2-5 are mitochondrial enzymes. In addition, the analysis of Glx2-1−YFP (yellow fluorescent protein) fusion proteins indicates that Glx2-1 is localized in the mitochondria (unpublished results). Subsequent biochemical studies confirmed that Glx2-2 and Glx2-5 catalyze the hydrolysis of SLG (*5*, *13*), and crystallographic and spectroscopic studies revealed that Glx2-5 contains an FeZn center (*5*). Glx2-3 was shown to bind a single Fe ion, but this protein did not hydrolyze SLG (*14*). Subsequent studies have suggested that Glx2-3 is in fact ETHE-1 (ethylmalonic encephalopathy protein 1) (*15*), and recent studies demonstrate that ETHE-1 is a mitochondrial sulfur dioxygenase (*16*). We have recently reported that Glx2-1 contains a dinuclear metal center; however, Glx2-1 does not exhibit glyoxalase 2 activity (*17*). The work presented here describes our efforts to identify a substrate for Glx2-1.

A. thaliana glx2-1 was cloned into pET26b, and the resulting pGlx2-1/pET26b plasmid was transformed into *Escherichia coli* BL21(DE3) competent cells (*17*). Glx2-1 was overexpressed in LB medium supplemented with 250 μM Fe(II) and Zn(II). The resulting, purified protein was >95% homogeneous (Figure S1) and was shown to bind 0.5 ± 0.3 equiv of Zn(II) and 1.4 ± 0.6 equiv of Fe. Consistent with previous results (*17*), it exhibited no glyoxalase 2 activity.

In an attempt to identify a Glx2-1 substrate, the purified enzyme was used in Merlin Micronaut TAXA PROFILE E assay plates, which allow for the rapid screening of 192 different substrates and several different reaction types. Analyses of the resulting assays revealed a large number of positive reactions. Therefore, we transformed *E. coli* BL21(DE3) cells with an empty pET26b plasmid, performed the purification protocol, and pooled and concentrated the fractions in which Glx2-1 would have eluted from the column. When this protein mixture (prep minus Glx2-1) was used in the Merlin assay plates, we found that nine reactions were uniquely catalyzed by purified Glx2-1. All of the potential substrates were nitrophenyl-substituted sugars: *p*-nitrophenyl α-L-arabinopyranoside, *o*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl β-D-lactopyranoside, *p*-nitrophenyl β-D-fucopyranoside, *p*-nitrophenyl α-L-arabinofuranoside, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-galactopyranoside, *o*-nitrophenyl β-D-galactoside, and *p*-nitrophenyl *N*-acetyl β-D-glucosaminide. However, when we attempted to confirm these substrates using a steady-state kinetic assay as a secondary screen, none of the substrates were hydrolyzed by purified Glx2-1. This seemingly contradictory result can be

†This work was supported by the National Institutes of Health (Grant GM076199 to C.A.M.), a Miami University/Volwiler Professorship (to M.W.C.), a Miami University Presidential Academic Enrichment fellowship (to P.L.), and the Robert A. Welch Foundation (Grant F-1572 to W.F.).

*To whom correspondence should be addressed. Phone: (513) 529-7274. Fax: (513) 529-5715. E-mail: crowdemw@muohio.edu.

Published on Web 08/17/2009

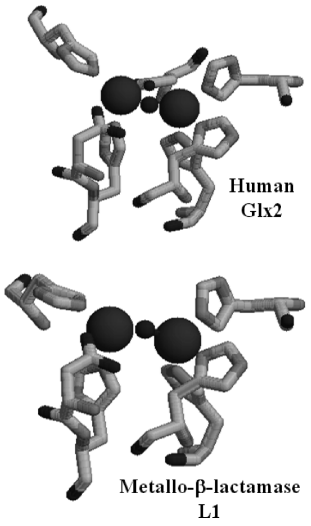

FIGURE 1: Active site structures of (top) human Glx2 and (bottom) metallo-β-lactamase L1. This figure was drawn using Raswin version 2.7.3 and coordinates from Protein Data Bank entries 1QH5 (for Glx2) and 1SML (for L1).

explained by the different reaction conditions of the Merlin reactions and the steady-state kinetic reactions. The Merlin kit reactions were conducted for 24 h at 37 °C, while the steady-state kinetic studies were conducted for 1−2 h at 25 °C. If Glx2-1 does in fact exhibit nitrophenyl-substituted sugar hydrolase activity, the rates are very slow.

Therefore, we tested whether Glx2-1 could hydrolyze substrates of other β-lactamase fold enzymes. The Merlin kit contained sulfatase substrates, so Glx2-1 does not exhibit arylsulfatase activity. We then tested whether Glx2-1 could hydrolyze *p*-nitrophenyl bisphosphate and *p*-nitrophenyl trisphosphate, which are substrates of zinc phosphodiesterase (ZiPD); however, neither compound was hydrolyzed by Glx2-1. We also tested whether Glx2-1 could hydrolyze lactones, such as γ-butyrolactone and a series of *N*-acyl-L-homoserine lactones (AHLs) that are commonly found in the rhizosphere, all of which are substrates for the related quorum-quenching AHL lactonases (*18*). However, Glx2-1 did not hydrolyze any of these compounds.

Although having only 10% overall sequence identity (Table S1), Glx2-1, like other Glx2 enzymes, most closely resembles metallo-β-lactamase L1 from *Stenotrophomonas maltophilia* in terms of metal binding ligands (*19*), except for the presence of an additional Asp in Glx2-1 (Figure 1). Therefore, we tested whether Glx2-1 could catalyze the hydrolysis of the amide bond in several β-lactam-containing antibiotics. Surprisingly, Glx2-1 exhibited a k_{cat} of 1.20 ± 0.04 s^{-1} and a K_m of 9.9 ± 1.6 μM ($k_{cat}/K_M = 1.2 \times 10^5$ M^{-1} s^{-1}) when using nitrocefin as the substrate. To test whether the observed hydrolysis was due to a constitutively expressed β-lactamase in *E. coli* BL21(DE3) cells, we assayed the prep minus Glx2-1 protein mixture for β-lactamase activity. The prep minus Glx2-1 sample exhibited essentially no hydrolysis activity against nitrocefin (Figure 2). As an additional control, purified point mutants (R172H, R172H/N174Y, and R172H/N174Y/Q249R/R252K) of Glx2-1 were also shown to be incapable of hydrolyzing nitrocefin at appreciable rates (R172H exhibited a k_{cat} value of 0.0023 s^{-1}, and the other mutants exhibited k_{cat} values ≤ 0.0005 s^{-1}). Lastly, column fractions for wild-type Glx2-1 and the R172H/N174Y mutant were assayed, and activity tracked with the presence of wild-type Glx2-1 (Figure S2). Glx2-1 was also used in assays to ascertain

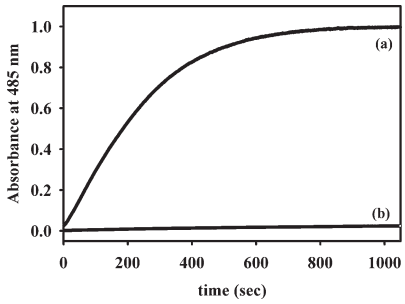

FIGURE 2: Nitrocefin hydrolysis by (a) Glx2-1 and (b) prep minus Glx2-1. The concentration of nitrocefin was 96 μM, and the buffer was 10 mM MOPS (pH 7.2). Hydrolyzed product formation was monitored at 485 nm.

whether the enzyme could hydrolyze cefotaxime or imipenem. The enzyme exhibited the following steady-state kinetic constants: $k_{cat} = 0.20 \pm 0.01$ and 0.20 ± 0.01 s^{-1}, and $K_m = 25 \pm 5$ and 23 ± 3 μM when using cefotaxime ($k_{cat}/K_M = 8.0 \times 10^3$ M^{-1} s^{-1}) and imipenem ($k_{cat}/K_M = 8.7 \times 10^3$ M^{-1} s^{-1}), respectively. The steady-state kinetic constants exhibited by Glx2-1 are significantly lower (10−100-fold lower k_{cat}/K_m values) than those typically exhibited by most purified metallo-β-lactamases (*20*), suggesting that β-lactams may not be the natural substrate of the enzyme. It is, however, interesting to note that Glx2-1 does exhibit higher k_{cat}/K_m values than the mononuclear Zn(II) containing A2 enzyme from *Aeromonas hydrophila* (*20*). In addition, Glx2-1 is >40-fold more active than the evolved metallo-β-lactamase, which was prepared by combining the scaffold of human Glx2 with loops and other structural components of metallo-β-lactamase IMP-1 (*21*). It is interesting that this in vitro evolution may have occurred in nature with Glx2-1.

To further probe the reaction of Glx2-1 with the β-lactam-containing antibiotics, stopped-flow kinetic studies were conducted. Previous kinetic studies revealed that most of the metallo-β-lactamases, which contain a dinuclear metal center, form a reaction intermediate, which absorbs at 665 nm, during the hydrolysis of nitrocefin, while the mononuclear Zn(II)-containing analogues and metallo-β-lactamase BcII [dinuclear Zn(II)] do not (*4*). The reaction of 15.5 μM Glx2-1 with 94 μM nitrocefin resulted in stopped-flow progress curves in which the substrate was depleted in 20 s (Figure S3). There was no buildup of species at 665 nm, as is seen with some metallo-β-lactamases. This result indicates that the active site of Glx2-1 is not conducive to stabilizing the ring-opened, nitrogen anionic intermediate of nitrocefin, suggesting that Glx2-1 has an active site more similar to that of BcII.

It is not clear why there should be β-lactamase activity in plant mitochondria. To the best of our knowledge, plants do not produce β-lactams, and β-lactams are not present in plant mitochondria. The plant pathogen *Erwinia carotovora* as well as *Serratia* and *Photorhabdus* species do produce carbapenem β-lactams (*22*), but these compounds presumably target competing bacteria. A recent bioinformatics study did identify a putative serine β-lactamase in plants, although the authors of this paper speculated that enzyme was in fact a catabolic serine protease (*23*). β-Lactam-containing antibiotics exert their antimicrobial properties by inactivating bacterial transpeptidases, which cross-link peptidoglycan building blocks in the cell wall. β-Lactam-containing antibiotics do not affect plant cells in the same way. In mammalian cells, there are reports indicating that cephalosporins are nephrotoxic (*24*), and the toxicity is apparently caused by either the inhibition of cytochrome *c* oxidase (*25*) or by

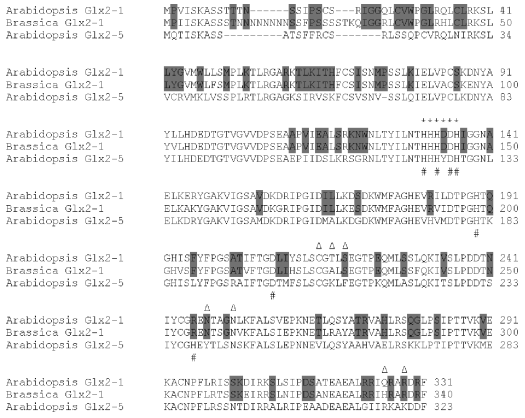

FIGURE 3: Alignment of predicted plant mitochrondrial Glx2-1 and Glx2-5 from *A. thaliana* and Glx2-1 from *Brassica*. The asterisks mark the β-lactamase fold, metal binding motif. The number signs mark the highly conserved metal binding residues, and the deltas mark the substrate binding residues of Glx2-5. Residues conserved in Glx2-1 but differing in Glx2-5 are highlighted in gray.

interference with carnitine transport in the mitochondria (*26*). It has been suggested that β-lactams can enter animal cells via the OCTN2 transporter (*27*). However, at this time, the role β-lactamase activity plays in plant mitochondria, if any, is unclear.

Database searching indicated a Glx2-1 homologue is not present in the completed genome sequences of other organisms, including those of rice and poplar, or in the available cDNA and EST sequence databases. We did, however, identify a potential Glx2-1 gene in the partial *Brassica* (Figure S4) and *Arabidopsis lyrata* genomic sequences (see Figure S5 for the phylogenetic tree). Primers based on the *Brassica* sequence were used in RT-PCR experiments to isolate a full-length cDNA for *Brassica* Glx2-1, which is shown aligned with *Arabidopsis* Glx2-1 and Glx2-5 in Figure 3. Therefore, while Glx2-1 does not appear to be widespread in nature, it is found in *Arabidopsis* and related crucifers. We propose that Glx2-1 may represent an example of ongoing gene evolution (i.e., gene duplication and divergence of activity). Duplication and functional divergence of an ancestral mitochondrial Glx2 gene have led to the emergence of β-lactamase activity in Glx2-1. At this time, it is not clear if Glx2-1 hydrolyzes β-lactams in plant cells or if it has a different preferred substrate. Glx2 and many of the β-lactamases play protective roles in the cell; therefore, we predict that ultimately this may also be the case for Glx2-1.

Discovery of β-lactamase activity in a glyoxalase scaffold as well as the wide range of activities catalyzed by the metallo-β-lactamase superfamily (*1*) suggests that these proteins may be excellent templates for the evolution of new functions. However, the fact that the amidohydrolase superfamily (*28*, *29*) has an entirely different protein scaffold yet shares some mechanistic features and some of the same promiscuous activities with metallo-β-lactamase superfamily members suggests that the metal centers of these enzymes may serve as an inherently promiscuous catalytic unit that is refined and enhanced by changes to its local protein environment in response to selective pressures.

ACKNOWLEDGMENT

We thank Prof. Richard Moore of Miami University for his assistance in generating the phylogenetic tree.

SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Daiyasu, H., Osaka, K., Ishino, Y., and Toh, H. (2001) *FEBS Lett. 503*, 1–6.
2. Kawashima, Y., Ohki, T., Shibata, N., Higuchi, Y., Wakitani, Y., Matsuura, Y., Nakata, Y., Takeo, M., Kato, D., and Negoro, S. (2009) *FEBS J. 276*, 2547–2556.
3. de la Sierra-Gallay, I. L., Pellegrini, O., and Condon, C. (2005) *Nature 433*, 657–661.
4. Crowder, M. W., Spencer, J., and Vila, A. J. (2006) *Acc. Chem. Res. 39*, 721–728.
5. Marasinghe, G. P. K., Sander, I. M., Bennett, B., Periyannan, G., Yang, K. W., Makaroff, C. A., and Crowder, M. W. (2005) *J. Biol. Chem. 280*, 40668–40675.
6. O'Young, J., Sukdeo, N., and Honek, J. F. (2007) *Arch. Biochem. Biophys. 459*, 20–26.
7. Talesa, V., Rosi, G., Contenti, S., Mangiabene, C., Lupattelli, M., Norton, S. J., Giovannini, E., and Principato, G. B. (1990) *Biochem. Int. 22*, 1115–1120.
8. Oray, B., and Norton, S. J. (1982) *Methods Enzymol. 90*, 547–551.
9. Al-Timari, A., and Douglas, K. T. (1986) *Biochim. Biophys. Acta 870*, 219–225.
10. Cameron, A. D., Ridderstrom, M., Olin, B., and Mannervik, B. (1999) *Structure 7*, 1067–1078.
11. Limphong, P., McKinney, R. M., Adams, N. E., Bennett, B., Makaroff, C. A., Gunasekera, T. S., and Crowder, M. W. (2009) *Biochemistry 48*, 5426–5434.
12. Maiti, M. K., Krishnasamy, S., Owen, H. A., and Makaroff, C. A. (1997) *Plant Mol. Biol. 35*, 471–481.
13. Wenzel, N. F., Carenbauer, A. L., Pfiester, M. P., Schilling, O., Meyer-Klaucke, W., Makaroff, C. A., and Crowder, M. W. (2004) *J. Biol. Inorg. Chem. 9*, 429–438.
14. Holdorf, M. M., Bennett, B., Crowder, M. W., and Makaroff, C. A. (2008) *J. Inorg. Biochem. 102*, 1825–1830.
15. McCoy, J. G., Bingman, C. A., Bitto, E., Holdorf, M. M., Makaroff, C. A., and Phillips, G. N. J. (2006) *Acta Crystallogr. D62*, 964–970.
16. Tiranti, V., Viscomi, C., Hildebrandt, T., Di Meo, I., Mineri, R., Tiveron, C., Levitt, M. D., Prelle, A., Fagiolari, G., Rimoldi, M., and Zeviani, M. (2009) *Nat. Med. 15*, 200–205.
17. Limphong, P., Crowder, M. W., Bennett, B., and Makaroff, C. A. (2009) *Biochem. J. 417*, 323–330.
18. Thomas, P. W., Stone, E. M., Costello, A., Tierney, D. L., and Fast, W. (2005) *Biochemistry 44*, 7559–7569.
19. Ullah, J. H., Walsh, T. R., Taylor, I. A., Emery, D. C., Verma, C. S., Gamblin, S. J., and Spencer, J. (1998) *J. Mol. Biol. 284*, 125–136.
20. Felici, A., Amicosante, G., Oratore, A., Strom, R., Ledent, P., Joris, B., Fanuel, L., and Frere, J. M. (1993) *Biochem. J. 291*, 151–155.
21. Park, H. S., Nam, S. H., Lee, J. K., Yoon, C. N., Mannervik, B., Benkovic, S. J., and Kim, H. S. (2006) *Science 311*, 535–538.
22. Coulthurst, S. J., Barnard, A. M. L., and Salmond, G. P. C. (2005) *Nat. Rev. Microbiol. 3*, 295–306.
23. Liobikas, J., Baniulis, D., Stanys, V., Toleikis, A., and Eriksson, O. (2006) *Biologija,* 126–129.
24. Tune, B. M., and Hsu, C. Y. (1995) *J. Pharmacol. Exp. Ther. 274*, 194–199.
25. Kiyomiya, K. i., Matsushita, N., Matsuo, S., and Kurebe, M. (2000) *Toxicol. Appl. Pharmacol. 167*, 151–156.
26. Pochini, L., Galluccio, M., Scumaci, D., Giangregorio, N., Tonazzi, A., Palmieri, F., and Indiveri, C. (2008) *Chem.-Biol. Interact. 173*, 187–194.
27. Ganapathy, M. E., Huang, W., Rajan, D. P., Carter, A. L., Sugawara, M., Iseki, K., Leibach, F. H., and Ganapathy, V. (2000) *J. Biol. Chem. 275*, 1699–1707.
28. Roodveldt, C., and Tawfik, D. S. (2005) *Biochemistry 44*, 12728–12736.
29. Seibert, C. M., and Raushel, F. M. (2005) *Biochemistry 44*, 6383–6391.